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PROTEIN MONOLAYERS

by

G.W. Hodgson, B.Sc.

Department of Chemistry
University of Alberta

April 1947

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A Thesis

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G.W. HODGSON, B.Sc.

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Department of Chemistry.

April 19, 1947

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FACULTY OF ARTS AND SCIENCES

This is to certify that the undersigned have read and recommended to the Committee on Graduate Studies for acceptance, a thesis submitted by G.W. Hodgson, B.Sc., entitled

"Protein Monolayers"

Professor

Professor

Professor

PROTEIN MONOLAYERS

Submitted in Partial Fulfillment

of the

Requirements for the degree of

Master of Science

by

G. W. Hodgson, B.Sc.

Under the direction of

Dr. J.L. Morrison

Time devoted to this work - 10 months

Edmonton, Alberta April, 1947

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INTRODUCTION

INTRODUCTION

Proteins have long been recognized as having very complex chemical structures. Conventional chemical and physical methods have failed to yield a complete picture of protein structure. It is desirable to supplement such studies by data obtained from as many methods of attack as possible.

Monolayer work reported herein is primarily concerned with the determination of molecular weights of plant proteins. In addition to this, data are presented on other properties of protein monolayers, such as compressibilities, limiting areas, dipole moments, and expansion patterns. As the protein monolayer work is part of a larger project initiated to study plant proteins, monolayer data are correlated with osmotic pressure and electrophoretic measurements in an attempt to elucidate the structure of proteins in general.

Molecular weights, and other properties, have been determined for egg albumin as a control, and for the three pea proteins: legumin, vicilin, and legumelin.

PROTEINS

Proteins are chemical compounds in which the alpha amino acid is the basic building unit. Fischer's hypothesis stated that the alpha amino acids are joined up by peptide linkages forming long chains. Bergmann and Niemann (6) concluded that certain simple numerical relations hold between the amino acids in any protein. An expression was developed for the number of amino acid residues per molecule; further it was implied that each amino acid occurs in the peptide chain at regular intervals. A somewhat different theory has been developed by Wrinch for globular proteins (4). In the Wrinch cyclol theory pairs of amino acids are linked together in piperazone rings which are arranged in the cyclol fabric. The cyclol fabric is considered to be folded into a cage-like structure forming globular proteins. Other theories of protein structure have been advanced also.

Protein molecular weights range from five thousand to several million. The methods of determining molecular weights are by osmotic pressures, sedimentation and diffusion, sedimentation equilibrium, size of unit cells in protein crystals (x-rays), viscosities, chemical analysis, and protein monolayers.

Although they are among the largest molecules known to science, protein molecules are very small. With Philippi (32) one might say that protein molecules are so small that the part played by their surface becomes altogether predominant. It is not surprising then to find many protein characteristics to be essentially surface characteristics.

THEORY OF MONOLAYERS

A monolayer or monomolecular film is a layer of material one molecule thick spread on a liquid or solid surface. The following survey will be limited to monolayers on liquid surfaces.

History

In 1890 Rayleigh observed that the surface tension of water was reduced by a layer of oil on the surface. Measurements carried out on such an oil film showed it to be one molecule thick. A short time later Pockels introduced the use of a trough with moveable barriers for the study of oil films. The next major advance was made by Langmuir in 1917 (20) who designed the first film balance to study oils and pure chemical compounds such as long chain fatty acids. Within the next few years the monolayer field was entered by many other workers: Adam and Harding, Schofield and Rideal, Schulman, Gorter, Harkins, Philippi and others. Monolayer technic has been applied to the study of proteins by Gorter, Langmuir, Philippi, Derivichian, Rideal, and very recently by Bull.

Monolayer Spreading

When a particular material is placed on the surface of a body of water it may do one of several things:

1. go into solution

2. pass through the surface unchanged

3. remain on the surface unchanged

4. spread out over the surface.

Three theories of spreading have been developed to explain the spreading process:

(a) Rayleigh's Theory: All liquids spread on the surface of clean water. An impurity on the surface will prevent spreading.

(b) Langmuir's Theory: The liquid will spread if molecules contain a polar group and a non-polar group.

(c) Harkin's Theory: This is a general thermodynamic theory covering all types of spreading (5). Briefly it states that a monolayer is formed with or without the formation of an intermediate unstable duplex film if the free energy of the film system is less than that of the clean water surface. A duplex film is described as one sufficiently thick that the upper and lower surfaces of the film are independent of each other, and yet thin enough that properties of the film are independent of gravitational forces.

By definition, the lowering of the free energy of the surface is termed the film pressure. Any substance giving rise to such a pressure is said to be surface active. Since surface tension is a measure of the free surface energy, the film pressure may be expressed as the difference in surface tension

between clean water and the film-covered water.

Structure of the Film

Since the duplex film is known to exist only as an unstable intermediate film if at all, the discussion will be restricted to monomolecular films.

A monolayer may be pictured as molecules of the surface active material floating about on the surface. Langmuir's theory may be expanded here by saying that this system will be stabalized if the molecules are part non-polar and part polar; non-polar to make them insoluble in the underlying water, and polar to prevent them from evaporating if they are small molecules, and to prevent them from coalescing if they are large. The molecules are thus anchored to the surface where they are quite free to move about. An example of such a surface active material is a long chain fatty acid with its polar carboxyl group at one end of a non-polar hydrocarbon chain.

A study of such a simple arrangement of molecules on the surface should yield much information about size, shape, and other properties of the particles. Since the molecules are "anchored" to the surface the film is a two dimensional system. Matter in three dimensional systems can exist in three principal states, gaseous, liquid, and solid. It is reasonable to expect similar states of matter in two dimensional systems. Such is found to be the case. In addition to these three, several

additional states occur as shown in figure 1 (1). This variation is attributed to large lateral adhesional forces in the film.

Gaseous Films and Molecular Weights

Similar to an ideal gas, an ideal gaseous film is one consisting of molecules of negligible size without adhesion for each other. For an ideal gas the equation of state is

$$PV = nRT$$

This becomes $FA = nRT$ in two dimensions. The proof for this relation follows exactly that of the equation for three dimensions (3). By measuring the film pressure F, area A, temperature T, and knowing the gas constant R, one can calculate the number of moles "n" in a given film. Knowing the amount of surface active material comprising the film, the molecular weight can be readily calculated.

Real gases show deviations from the equation of state for an ideal gas a illustrated in figure 2. At constant temperature the product PV, for an ideal gas is constant, while for a real gas it is a function of the pressure. Gaseous films show similar deviations from the perfect gaseous film. At very low pressures the properties of a real gas approach those of an ideal gas as shown in figure 2.

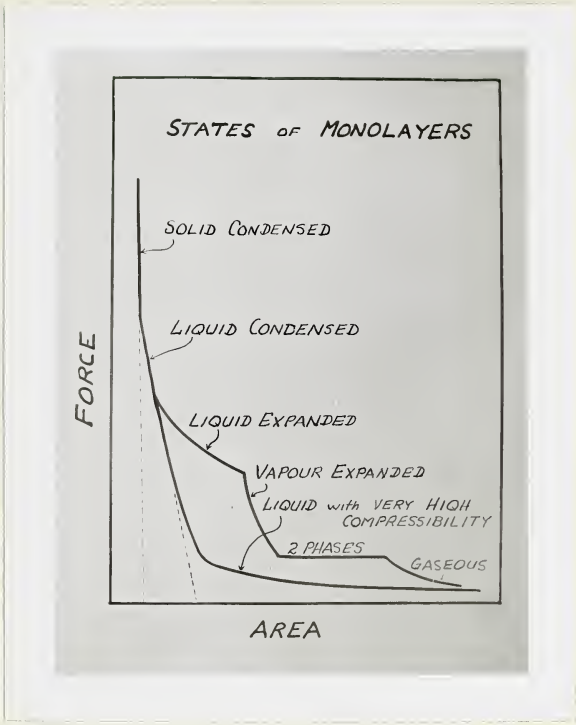


Figure 1.
States of Monolayers

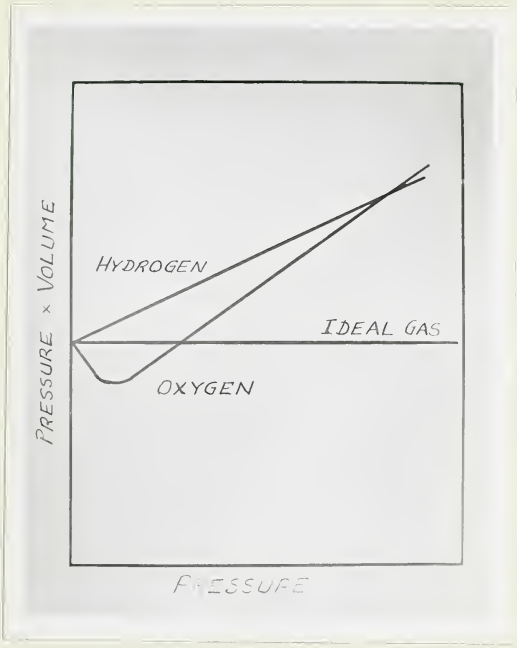


Figure 2.
PV-P Curves for Gases

In practice, film is prepared and film pressure readings F are made for corresponding film area readings A, as the film is compressed by reducing the area in steps. The FA-F curve is plotted from these readings and the equation for best straight line through the points is calculated. Fatty acid films are of oxygen type. All gaseous protein films studied have been found to be of the hydrogen type with an equation of the general form,

$$FA = a + bF$$

where a and b are constants.

When $F = 0$

$$FA = a$$

Since at very low pressures, the film becomes ideal the FA - intercept a may be used in the equation of state for an ideal gaseous film and the molecular weight calculated.

The slope b of the FA-F curve is quite important as it is a measure of the area of the surface actually covered by protein molecules, and hence can be used to calculate the dimensions of the molecules on the surface.

High Pressure Measurements - Condensed Films

In a gaseous film the molecules are free to move about with a considerable mean free path. When the film is compressed the molecules are pushed together. From an examination of the force-area relationships in the latter region

much can be learned about the nature of the molecular packing. Compression may be simply a matter of pushing the molecules together on the surface until they are as closely packed as they are in a crystal. On the other hand the molecules on the surface may be extensively re-orientated during compression giving rise to the expanded type of film.

As suggested in figure 1, an extrapolation to zero force of the condensed portion of the F-A curve gives a value for the limiting area of the material on the surface. If re-orientation takes place during compression one might find two or more limiting areas. A comparison can be made between the high pressure limiting areas and the area obtained from the slope of the FA-F curve for the gaseous region.

A plot of the compressibility coefficient $-\frac{1}{A}\frac{dA}{dT}$ against the film area is useful in the study of condensed films. Such a curve describes in detail the behavior of the film on compression. The shape of such a curve and the point of minimum compressibility can be used empirically in the identification of unknown materials.

Surface Potentials

By definition, the surface potential of a film is the difference between the potential existing across a clean water surface and that across the film-covered surface. As Langmuir

suggested in his theory of spreading, the surface active molecule is highly orientated at the surface with the polar group down in the water and the non-polar part away from the surface. This indicates that the molecule on the surface exists as a dipole. As such it would profoundly influence the potential difference across the water surface. As compression of the film is carried out the molecules of the film are pushed closer together thus causing a steady change in the surface potential of the film. If the potential is expressed as charge per molecule which is in effect the proportional to the dipole moment of the molecule, a change would not occur until the orientation of the molecule itself was altered. The term "orientation" is used here to cover any change in the nature of the molecules in the film.

Helmholtz (4) showed that the change ΔV in surface potential caused by a film containing n molecules per square centimetre is given by

$$\Delta V = 4\pi n\mu$$

is the average vertical component of the change in dipole moment of the part of the surface occupied by a film molecule, including all effects due to re-distribution of ions in the interior of the solution, and re-orientation of water molecules. Schulman and Rideal (4) assumed that the term μ could be split up into two completely separate parts; $\bar{\mu}$ expressing the

absolute vertical component of the dipole of each film molecule and another term due to redistribution of ions and re-orientation of the water molecules when the film is formed. Adam and Harding (4) showed that this assumption was invalid and favoured the use of μ as an indication of the orientation of the dipoles in the film.

A change in molecular orientation should be accompanied by a change in dipole moment of the molecules.

$$\Delta V = 4\pi n\mu$$

For a given film n is inversely proportional to the area. Then

$$\Delta VA = 4\pi\mu k'$$

$$\Delta VA = k\mu$$

where k and k' are constants. The relation between the vertical component of the dipole moment and the area may be shown in a VA-A plot.

In addition to this, surface potential measurements are indispensible for determining the homogeneity of films. Widely varying potentials from point to point on the surface indicate a multi-phase system while constant readings indicate a homogeneous film.

Expansions Patterns

A very simple technique for the study of protein monolayers has been developed by Schaefer (33). An indicator oil is prepared by mixing oxidized lubricating oil with pure mineral oil. The oxidized oil makes the mixture surface active to form a duplex film. The proportion of oxidized oil to mineral oil in the mixture is such that the oil film gives the first order yellow interference color on compression to one dyne*. A small drop of the oil is placed on a clean water surface where it spreads radially. The amount of oil so applied is enough to cover about half the available area. A small amount of protein is applied to the surface at the same point as the oil was placed. The protein spreads at very low pressure and pushes the oil ahead of it. The oil-protein boundary is clearly visible, and is called the external spreading boundary. The films are then compressed until the indicator oil shows a pressure of nearly one dyne. A second application of indicator oil is made, this time in the centre of the protein monolayer. The protein film is ruptured in a manner characteristic of the particular protein used. The external spreading boundary and the latter internal boundary comprise the expansion pattern.

The expansion pattern is an indication of the state of the monolayer at that particular pressure. A gaseous or liquid

* 'Dynes' will often be used for dynes per centimetre.

film is characterized by a smooth circular pattern. A star-shaped internal boundary is obtained from a rigid condensed film. The external boundary is less useful than the internal because it is a function of the rate of spreading, a variable that is difficult to control in practice.

Although the choice of one dyne may seem arbitrary, it is a critical pressure for protein films, in that the films of most proteins undergo a change of state in that region.

Related Methods

In the discussion of the protein monolayer results, comparisons will be made with the osmotic pressure and electrophoretic measurements made by W.B. McConnell (27) and L. Wetter (26) respectively. A brief outline of these methods is useful at this point.

Osmotic Pressure:

The molecular weight of a protein can be determined from the osmotic pressure of its solution. The weight so obtained is a number average as is the monolayer molecular weight.

Electrophoresis:

A protein molecule in solution in an electric field tends to migrate because of the charge on the molecule. Electrophoresis studies are concerned with the manner in which a protein-buffer boundary moves under such conditions. Molecules of

different protein species have different charge-to-mass ratios and hence the rates of migration are different. A homogeneous protein in solution is characterized by a single peak in the protein boundary, while a heterogeneous solution may have several peaks. Relative sizes of the peaks are an indication of the relative concentrations of the protein fractions in solution. By means of a suitable optical system the boundary can be photographed.

APPARATUS

APPARATUS

The basic unit of surface film apparatus in a long, narrow, shallow tray filled to the brim with water or some aqueous solution on which the film is spread. The area of the film is restricted by suitable barriers placed across the tray at both ends. Measurements are then made on the film by:

(a) horizontal film balance

(b) Wilhemly (vertical) film balance

(c) Surface potential system.

These measurements may be made simultaneously on the same film. This apparatus is described in great detail by Hahn (15). A brief summary with modifications follows.

(a) Horizontal Film Balance

The horizontal film balance of the Langmuir-Adam type used in this work was that designed and built by J.W.V. Hahn (15). Figure 3 illustrates its operation. The basic unit is the long narrow tray filled with a suitable aqueous substrate. Near one end is a mica barrier F, floating on the surface, joined to the edges of the tray with thin (0.01 mm.) S-shaped gold ribbons G which serve to confine the film while still allowing the float to move freely within limits. The film is confined at the other end of the tray by a glass barrier B. When the monolayer is compressed by the glass barrier it exerts an increase in

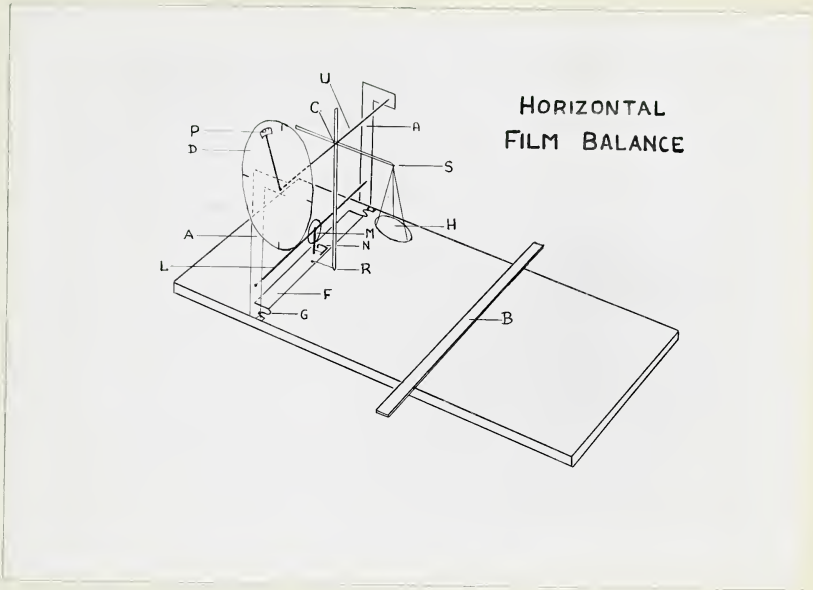


Figure 3

pressure against all its boundaries. The only moveable part of the boundary is the mica float. Its motion activates the small concave mirror M, on the lower torsion wire L. Thus a change in film pressure is registered by a change in the position of the spot of light reflected from the mirror to the scale some seven feet away. A torque is then applied to the upper torsion wire U to return the light to its original zero position. The amount of torque is registered on the dial D by the position of the pointer P attached directly to the upper torsion wire. Thus dial readings are directly proportional to the film pressure. A calibration to find the proportionality factor can be made by placing weights on the small pan H hung from the horizontal arm and determining the dial readings necessary to return the light to its original position. The horizontal film balance is used as a null instrument.

The sensitivity of this balance can be altered by altering the position of the weight on the vertical arm to which the float is linked. The mirror is mounted securely on a separate torsion wire (frictionless bearing) rather than on the main torsion wire in order to magnify displacements of the float.

The sensitivity of the balance was increased by changing the positions of the gold ribbons to allow greater

freedom of movement of the float. It was felt that the greater sensitivity so obtained overshadowed the degree of uncertainty introduced into the determination of float length.

The range of the instrument can be varied by changing the size of the upper torsion wire. For all the work reported here a phosphor-bronze wire, 0.127 mm. in diameter was used. The range of pressures measurable with this wire is 0.005 to 1 dyne per centimetre.

(b) Wilhelmy Film Balance

The vertical film balance measures changes in surface tension by changes in the pull on a glass slide dipping through the surface of the water. This method is described in detail by Harkins and Anderson (16).

Figure 4, shows the principle of the operation of the Wilhelmy film balance. With the slide suspended in clean water the weights on the pans are so adjusted that the slide hangs low, i.e. the light is high on the scale. When a film is placed on the surface the surface tension of the water is reduced so that the slide is pulled out of the water and the spot of light moves down the scale.

It is found that

$$\Delta\gamma = \frac{k\,\Delta S}{2(t+w)}$$

where $k = -g\,\frac{\Delta G}{\Delta S}$

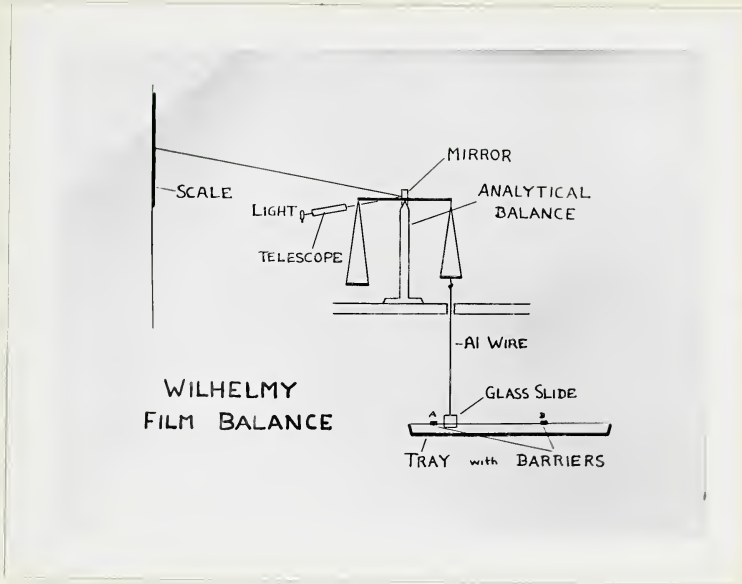

MIRROR
SCALE
LIGHT
ANALYTICAL BALANCE
TELESCOPE
-Al WIRE
WILHELMY FILM BALANCE
GLASS SLIDE
A
B
TRAY with BARRIERS

Figure 4

k, the proportionality factor is determined by finding the change in scale reading ΔS for known changes in weights on the pans ΔG. g, is the acceleration due to gravity; t and w the thickness and width of the glass slide; $\Delta\gamma$, the film pressure.

The Wilhelmy balance is not used as a null instrument.

The range of the instrument is determined by the sensitivity of the analytical balance and by the dimensions of the slide used. Two slides were used, one a microscope slide with a range of one to sixteen dynes and the other thicker glass, 2" square, giving three to forty dynes/cm. Thus the vertical balance was used for high pressure and the horizontal for low pressure measurements.

(c) Surface Potential System

By definition the surface potential of a monolayer is the difference between the potential drop across a clean water surface and the potential drop across a film covered surface.

A diagram of the circuit for measuring surface potentials is shown in figure 5. The main parts are the polonium coated air electrode which is so mounted that it can be used to explore the whole surface, and the FP-54 electronic tube amplifying circuit not shown. The aluminum tray was given about ten coats of Bakelite lacquer to provide electrical insulation of at least 5000 ohms.

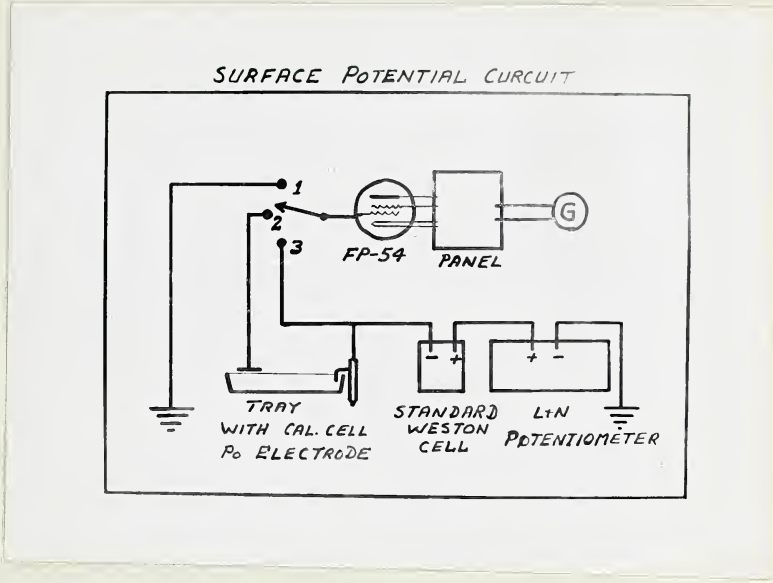

SURFACE POTENTIAL CURCUIT
1
2
3
FP-54
PANEL
G
TRAY
WITH CAL. CELL
Po ELECTRODE
STANDARD
WESTON
CELL
L+N
POTENTIOMETER

Figure 5

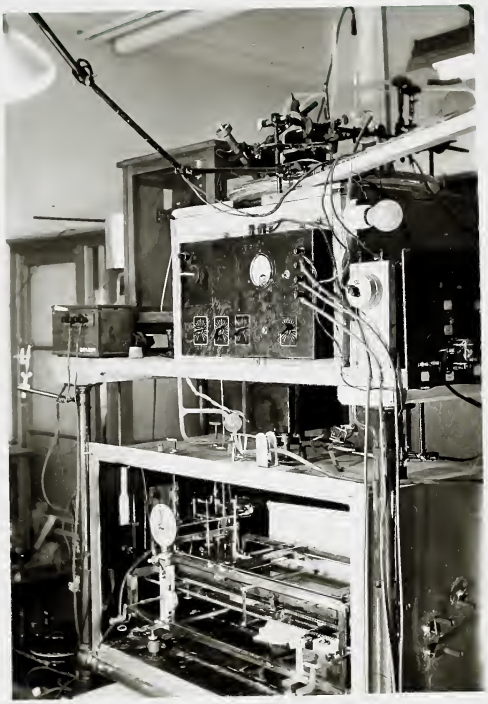
General Assembly

The horizontal balance is mounted on a concrete block set on a layer of cork. The balance is surrounded by a double wall copper box through which thermostated water is pumped. Balance controls are so arranged that the balance can be operated from outside the box. The polonium electrode is mounted in a carriage on the balance frame and the Wilhelmy slide is hung by a wire passing through the thermostating box from the pan of an analytical balance mounted above the box. Thus the film can be studied under conditions of constant temperature and high humidity. The high humidity is desirable for protein work in general. It also facilitates the use of the Wilhelmy balance by preventing excessive drying of the slide.

(d) Preparation of the Monolayer

The need for extreme cleanliness cannot be over emphasized. This is particularly true when low pressure measurements are to be made because errors in pressure measurements introduced by the presence of surface contamination are of the same order of magnitude as the pressures being measured. This will be discussed later.

The tray is cleaned by scrubbing it under a hot water tap, and then a very thin layer of paraffin is applied by painting a benzene solution of wax on the hot tray. The tray is allowed to stand over night and is then washed with a jet



General Assembly

of cold tap water, followed by distilled water.

The liquid on which the protein is spread is a 35% solution of ammonium sulfate as recommended by Bull (8). The use of a concentrated salt solution as the underlying solution makes the spreading of proteins into surface monolayers much more rapid and complete. Indeed some proteins which won't spread at all on water spread very readily on 35% ammonium sulfate solution. Reagent grade ammonium sulfate is dissolved in conductivity water. The solution is treated twice with norite (activated carbon) to remove any surface active contaminants.

Compression of the "clean" ammonium sulfate solution surface to one quarter the original area gives a pressure of about 0.03 dyne. It appears impossible to reduce the surface contamination below this point. The "residual contamination" may be due to a two dimensional vapour pressure of the paraffin on the edges of the tray.

The tray is then put in position and filled with the solution. Water is allowed to stand in pools on the bottom of the thermostat box as well as saturating a cheesecloth drape hung across the back of the box. Often steam is blown into the chamber to raise the humidity rapidly. The system is thermostated over night in order to attain a high humidity

and temperature equilibrium. The temperature gradient across the surface is measured by a twelve junction thermocouple so arranged that one series of junctions is in the ammonium sulfate solution while the other series is in the air beside the tray. Temperature differences are measured by a Type K potentiometer capable of detecting differences of less than 0.01°C. A temperature gradient of less than 0.05° is considered permissible.

Description of a Run

The surface is cleaned by about ten sweepings with paraffined glass barriers. The horizontal balance head is then lowered into position, the Wilhelmy slide installed and the clean-surface potential read. After about five minutes the zero positions of the balances are read and the protein is spread.

In general, the protein solution has a concentration of about five milligrams per cent nitrogen. The preparation of the protein solutions is given in a later section. The Blodgett micropipet (7) used to transfer an accurately measured volume of solution to the surface is shown in figure 6. It delivers 0.0785 millilitres of solution.

As the contents of the pipet are delivered to the surface spreading takes place rapidly. Film pressure and surface potential are read after equilibrium is attained. Usually two

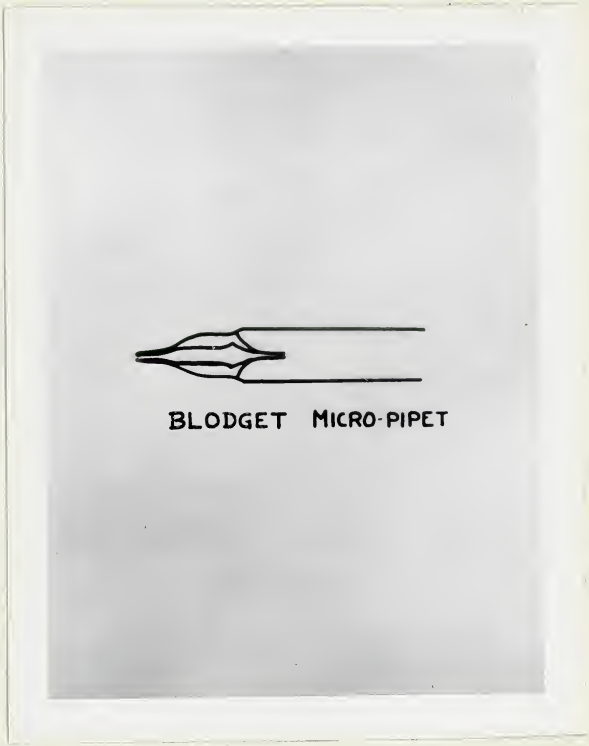


Figure 6

or three minutes are required to attain equilibrium in the low pressure range. The film is compressed further giving a series of pressure and potential readings. The electrode is moved about from time to time to check the homogeneity of the film. The Wilhelmy balance with the heavy glass slide begins to register a pressure as the range of the horizontal balance is exceeded at one dyne pressure. The float of the horizontal balance is then clamped in position and the run continued. In the higher pressure ranges, above fifteen dynes, ten or fifteen minutes are required for the film to come to equilibrium after each compression.

It is found that after five or six runs the residual contamination has increased about three fold. At this time the balance is dismantled for cleaning. The ammonium sulfate solution is either discarded or treated with norite.

Expansion Pattern Apparatus

Little equipment is required for the production of expansion patterns. A pyrex tray with ground edges and two or three glass barriers are the essentials. The edges of the tray and the barriers are paraffined. The indicator oil may be applied with a platinum wire and the protein with a stirring rod or pipet.

If a photographic record of the patterns is to be made the bottom of the tray should be painted black. The camera and lights are so arranged that high contrast between the oil and protein films is obtained.

PROTEIN PREPARATIONS

PROTEIN PREPARATIONS

(a) Egg Albumin

Egg albumin is the classical example of a protein of animal origin. Due to the fact that it can be readily prepared in a comparatively pure condition through a few recrystallizations it has been studied by many investigators. Thus the properties of this protein are fairly well established.

Egg albumin used in this work was prepared by the method of Keckwick and Cannan (20). It was recrystallized three times and dialysed against distilled water until sulfate free. The solution was then diluted with conductivity water which had been treated with norite (activated carbon black). The concentration of the resulting solution was obtained by microkjeldahl nitrogen analysis using a potassium sulfate, selenium, mercuric oxide catalyst. All calculations involving egg albumin were based on a nitrogen content of 15.1% (9).

(b) Plant Proteins

Much of the early work on plant proteins was done by Osborne, 1891-1919 (30). Various plant globular proteins were crystallized by dialysing salt solutions against water (32). The globulin separates out either in crystals or spheroids, or as a mixture of both. Octahedral crystals were obtained from brazil nuts, hemp seed, flax seed, oats and squash seed but

none could be obtained from the legumes. Osborne and Harris found the pea (Pisum sativum) contained three proteins (33):

1. Legumin, a globulin, soluble in saline

2. Vicilin, a globulin more soluble in dilute saline. Vicilin solutions coagulated on heating to a boil; legumin solutions remained clear at 100°.

3. Legumelin, an albumin with properties quite distinct from the other two.

The method of separation used by Osborne is as follows: A ten per cent sodium chloride extraction of pea meal is saturated with ammonium sulfate precipitating the vicilin and legumin. The precipitate is redissolved in dilute ammonium sulfate and dialysed for five days. The precipitated legumin and vicilin are dissolved again in ten per cent salt, filtered and dialysed ten days. To the dialysed solution is added enough ammonium sulfate to make it six-tenths saturated. The precipitated legumin is dissolved in dilute ammonium sulfate solution and dialysed seven days. The vicilin is precipitated by increasing the ammonium sulfate concentration to eight tenths. The vicilin is redissolved and precipitated by dialysis for seven days. The legumelin is made up of two parts, one precipitated by the addition of acetic acid to the saturated salt solution while the other remains in solution (31).

Peptization studies of pea flour (14) showed that there was a peptization maximum at 10-20% and a less distinct one at 2% NaCl. Presumably these maxima corresponded to the legumin and vicilin of Osborne.

The isoelectric points for vicilin and legumin have been found to be 5.2 and 5.35 respectively (12).

Modifications of Osborne's Method

Osborne's method of fractionating pea proteins involved long periods of dialysis. This was undesirable because there are proteolytic enzymes very closely associated the pea proteins which bring about changes in the protein structure. The following procedure was developed to shorten the period of preparation of the pea protein fractions.*

The peas were balled milled for several hours to give a very finely divided meal. About 90% of the total protein was extracted in a single 10% NaCl extraction. A subsequent water extraction removed about 8% more. The pH of the saline extract was changed from 6.0 to 5.25 by the addition of dilute acetic acid. A dilution of one to five precipitated most of the legumin which was redispersed in 10% NaCl and reprecipitated

* In this preparation as in all subsequent preparations of pea proteins all precipitations were made at a pH of 5.2. Normal acetic acid was used to adjust the pH whenever necessary. Measurements of pH were made with the Coleman pH Electrometer.

several times. The supernatant liquid from the dilution contained all the vicilin and the remainder of the water soluble legumelin. To precipiate the vicilin, the salt concentration was reduced by dialysis rather than further dilution in order to keep the volume of solution down. The supernatant was dialysed one day and the pH adjusted to 5.25 and the vicilin centrifuged down.

The legumin obtained after several purifications accounted for about six per cent of the original protein; the legumelin 15%; and the vicilin six per cent.

Preparation of Vicilin

It was felt that the above fractionation could be improved by the use of an electrodialyser to eliminate the dilution step:

1. 40 gm. of ball milled pea meal were extracted for several hours with 500 ml. of 10% NaCl solution, followed by a second extraction with 400 ml. 10% NaCl. The combined extracts were electro-dialysed to reduce the salt concentration to 2.6% to precipitate the legumin. NaCl concentrations were determined by titration with standard silver nitrate solution and potassium chromate indicator. For a complete flow sheet of this preparation see figure 7.

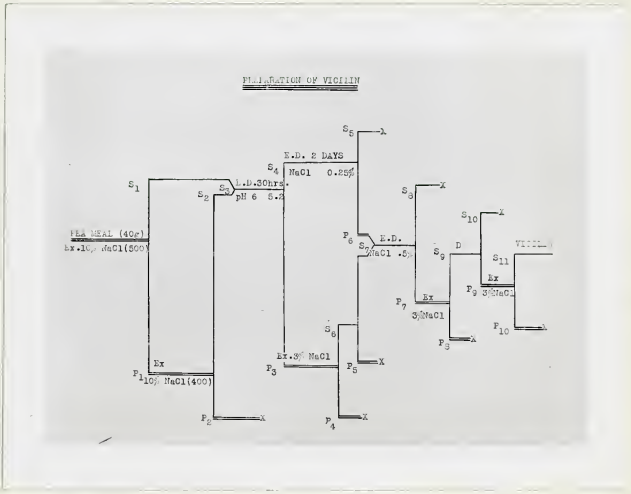


Figure 7
Preparation of Vicilin

In the flow sheet all vertical lines represent separations by centrifuging into the supernatant designated by a single horizontal line and the precipitate by a double horizontal line. X indicates discarded material. Centrifuging was done with a small angle-centrifuge at about 4000 r.p.m.

2. The supernatant liquid containing the legumelin and vicilin was further electrodialysed. Reducing of the salt concentration to less than one per cent precipitated the bulk of the vicilin.

3. Vicilin which was co-precipitated with the legumin was recovered by electrodialysing the crude legumin dissolved in 10% NaCl. This precipitated the legumin and left the vicilin in solution.

4. The combined vicilin extracts were then subjected to further purification by two precipitations and finally dissolved in 3% NaCl solution.

For monolayer work this solution was diluted with 3% NaCl solution which had been treated with norite.

W.B. McConnell and L. Wetter made osmotic pressure and electrophoresic runs respectively on the 3% stock solution of vicilin.

Preparation of Legumin (a)

This method of preparation had to be adopted because during prolonged electrodialysis of legumin solutions, the pH rose to 9.5 rendering the legumin quite insoluble in 10%-15% NaCl.

1. 40 gm. pea meal were stirred with 200 ml. of 10% NaCl for 15 minutes. 100 ml. were decanted off and another 100 ml. of NaCl were added to the meal. This was stirred for 45 minutes.

2. The suspension was centrifuged at 4000 r.p.m. in an angle centrifuge. The supernatant liquid was filtered through glass wool yielding 270 ml. of solution.

3. The pH was adjusted from 6.5 to 5.2. When the solution was diluted to two litres a white precipitate appeared. This was allowed to settle out overnight in the ice box. The cloudy supernatant liquid was decanted off.

4. The precipitate from #3 was redispersed in 150 ml. of 10% NaCl and centrifuged. Most of the material did not go into solution.

5. The supernatant from #4 was diluted to 750 ml. and allowed to stand over night in the ice box. The pH was read at 5.2.

6. The next morning a thin layer of material had settled out from a cloudy solution. The whole solution was centrifuged. The precipitate so obtained seemed quite soluble

in 25 ml. 10% NaCl although subsequent centrifuging precipitated most of it again.

7. The precipitate from centrifuging in #6 was stirred with 15 ml. of 10% NaCl and centrifuged again. This extract combined with that of #5 made up the legumin stock solution.

Monolayer runs were made on the stock solution of legumin, diluted to about 0.03% protein concentration with 10% NaCl. L. Wetter also made an electrophoretic run on this material diluted to 5% NaCl. Further dilution precipitated the legumin. This preparation is referred to as legumin (a) in later sections.

Preparation of Legumin (b)

The method of fractionation used here was essentially the same as that developed by Osborne. The extraction of the pea meal was made with phosphate buffer and the legumin precipitated by making the solution six-tenths saturated with respect to ammonium sulfate. The legumin was reprecipitated several times and finally dissolved in buffer solution.

An electrophoresis pattern was obtained for this material.

For monolayer work the solution was diluted with norite-treated conductivity water. Such dilution did not precipitate the protein.

Preparation of Legumelin

1. 80 gm. pea meal were stirred with 500 ml. distilled water for three hours.

2. The suspension was centrifuged and the supernatant was filtered through paper and dialysed against water for about eighteen hours.

3. The precipitate from dialysis was centrifuged down. The supernatant was saturated with ammonium sulfate and the precipitate was collected on a filter paper.

4. The precipitate was dissolved in water. The resulting solution was dialysed for two days. A considerable precipitate was filtered off. The filtrate was dialysed for 24 hours against 0.05 M NaCl. The presence of the salt was desirable in osmotic pressure measurements.

Monolayer runs were made on the dialysed solution diluted to about 0.03% protein with norite-treated conductivity water.

L. Wetter and W.M. McConnell made runs on the dialysed solution.

The concentrations of all protein solutions used in monolayer work were determined by microkjeldahl nitrogen analyses. Such analyses were carried out directly on the diluted (0.03%) protein solutions. All pea protein calculations are based on 17% nitrogen content as indicated by Osborne (33).

EXPERIMENTAL RESULTS

EXPERIMENTAL RESULTS

Molecular Weights

Egg Albumin

About forty-five molecular weight runs on egg albumin were made. The FA-F curve for each run was plotted. Three types of FA-F curves were obtained. These are illustrated by the plots of typical runs in figure 8. The first twenty-one runs were of the A type. Surface potential mapping indicated that the egg albumin did not cover the entire available surface of the tray. The egg albumin occupied the central portion of the tray. The boundary between egg albumin and surface contamination was made visible as shown in figure 9 by spreading the egg albumin in the midst of some lycopodium talc which had been dusted onto the surface. The value for FA in the FA-F plot is the product of the force exerted by the film and the area of the film as given by the area counter dial of the balance. The area counter dial is geared to the worm by which the moveable barrier of the balance is activated. Thus the value for A in the FA-F curve is too high when the egg albumin does not cover the whole surface. An area correction based on visual observation in talc runs lowered all the FA-F points. The points at the low pressure end were lowered by as much as fifty per cent. The correction factor decreased to about ten or fifteen per cent as the pressure was increased to 0.3 dynes and still less

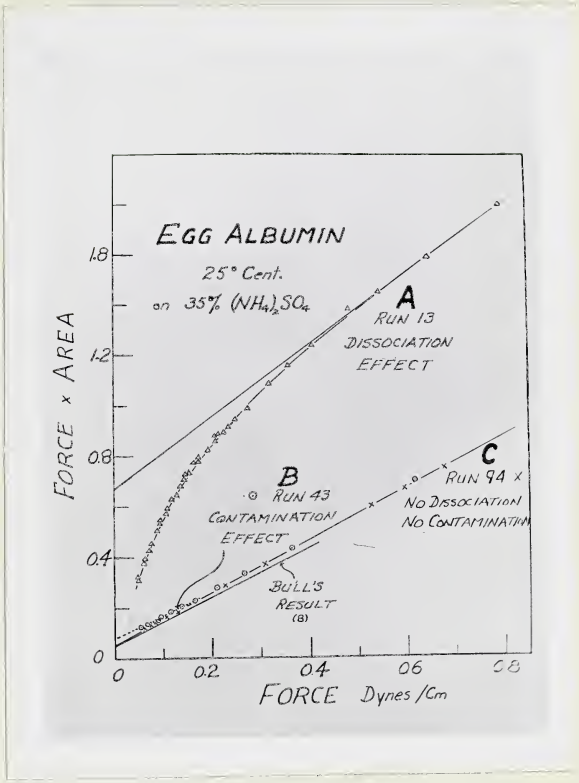


Figure 8
FA-F Curve for Egg Albumin

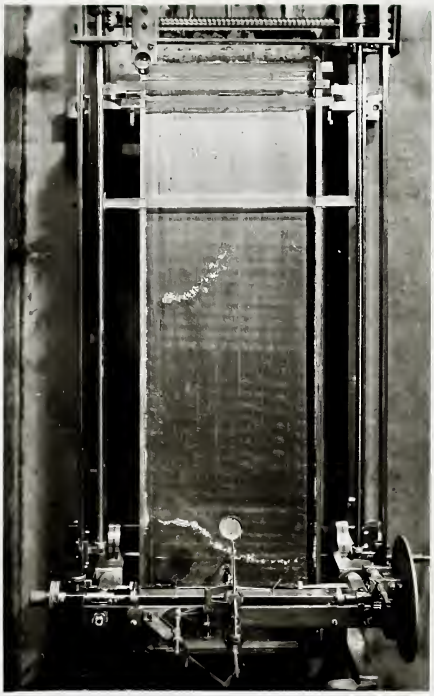


Figure 9.
Film Balance with Talc Boundary

at higher pressures.

Thus the shape of the FA-F curve was not appreciably changed by an area correction. It was found that the benzene solution of paraffin used to paraffin the edges of the tray was the main source of the contamination. When this solution was renewed the type A curve was still observed. Anomalous FA-F curves of type A were not caused by surface contamination.

An area correction applied to a type B run in Figure 9 changes it to a type C. In a type C run the points all lie on a straight line whereas a B run has a break at a pressure of about 0.2 dynes. Above this pressure the contamination is negligible. The improved technique for cleaning the tray described in a previous section was adopted late in the investigation. Most of the runs from then on were of the C type. Only when several consecutive runs increased the residual contamination were type B curves observed.

Figure 10 shows the distribution of molecular weights obtained from egg albumin at various initial surface concentrations. Very low surface concentrations were used in the early runs in order to get extremely low film pressure readings. Such readings would give FA-F points very close to the FA intercept from which the molecular weight is calculated. Thus additional accuracy could be attained by

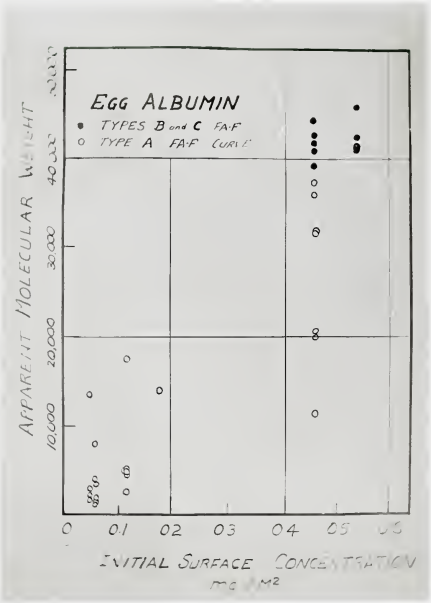


Figure 10.
Molecular Weight Distribution for
Egg Albumin

the use of the horizontal film balance at pressures below the range of the best Wilhelmy balance.

At low surface concentrations anomolous type A curves are obtained. From such curves low molecular weights are calculated. It is reasonable to suggest that dissociation of the egg albumin molecules takes place under these conditions.

To test the dissociation hypothesis of egg albumin monolayers the following runs were made. A protein filmwas spread at an initial concentration of 0.5 mg./square metre and compressed to a pressure of about 0.4 dynes. The underlying solution was then cooled by circulating cool water through a glass coil laying immersed in the solution on the bottom of the tray. The solution was cooled about five degrees and then warmed so it was about five degrees warmer than the thermostated air surrounding the balance. The solution was then cooled to its original temperature. The film pressure at the end of the run was found to be about twice the original pressure as shown in figure 11. A second run was made in which the order of heating and cooling was reversed. Again the pressure was approximately doubled. From an examination of the curves in figure 11, it appears that heating the underlying solution has a more immediate effect on the film pressure than cooling

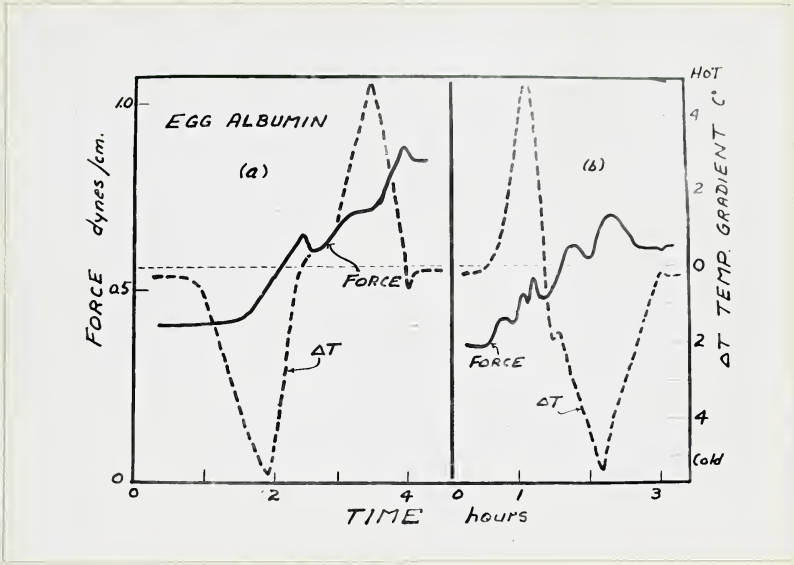


Figure 11.
T Curve

has. The doubling of film pressure indicates the presence of twice as many protein particles on the surface. This supports the dissociation hypothesis.

Thus when egg albumin is spread at concentrations above 0.4 mg. per square metre and zero temperature gradient, a straight line is obtained from the FA-F plot. By the method of least squares the equation of the line is found to be

$$FA = 0.0569 + 1.05\ F$$

where F is the force in dynes per centimetre and A the area of the film in square metres per milligram of protein. At zero force the molecules may be considered to be point particles with no attraction for one another; that is, the film is analagous to a perfect gas in three dimensions. It has been shown that for one mole of such a film at 25°C,

$$FA = RT$$
$$= 8.31 \times 10^7 \times 298 \text{ ergs}$$
$$= 24.7 \times 10^9 \text{ ergs}.$$

When F = 0,

$$FA = 0.0569 \frac{M^2}{mg.} \times \frac{dyne}{cm.}$$
$$= 0.0569 \times 10^4 \text{ ergs/mg.}$$

Thus the molecular weight is

$$\frac{24.7 \times 10^9}{0.0569 \times 10^4} = 4.32 \times 10^7 \text{ mg.}$$
$$= 43{,}200 \text{ gm.}$$

Vicilin

Vicilin is the pea globulin soluble in 3% NaCl solution. The FA-F curve was found to be a straight line with the equation

$$FA = 0.0860 + 0.81\ F$$

The molecular weight calculated from the extrapolated value of FA is 28,400. The mean deviations of thirteen runs indicates an error of about 1.8%. Dissociation variation was not noticed although initial surface concentration was varied over the wide range of from 0.26 to 1.05 mg. per square metre. All the runs were made at 25°C.

Legumin (a)

Legumin is the pea globulin soluble in 10% NaCl solution. Twenty-one molecular weight runs were made. The results are tabulated in table 1. Runs were made at six different temperatures; changes in absolute temperature had little or no effect on the observed molecular weight. Type C runs in which contamination was negligible show no better agreement than types A or B. Type A runs in which dissociation is believe to be taking place do not consistently give lower molecular weights than C runs as was the case with egg albumin. The conclusion is drawn that dissociation has probably taken place in all the runs. In types B and C the dissociation is complete before the pressure-area measurements are started

while in A the dissociation is incomplete at the start of the compression.

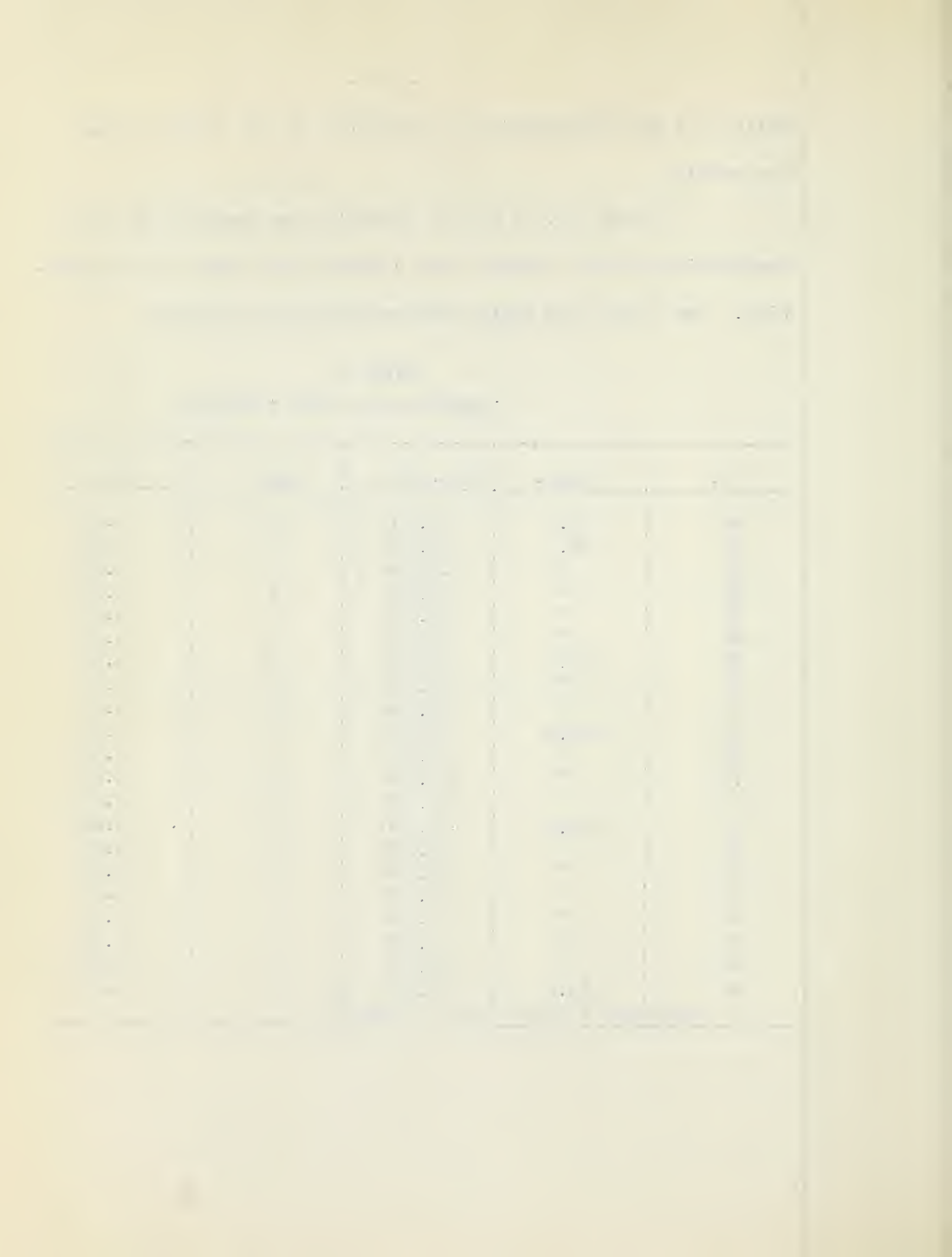
In runs 76, 77 and 78 spreading was carried out on a comparatively small surface thus reducing the chance of dissociation. The result was higher observed molecular weights.

Table I

Legumin (a) on 35% $(NH_4)_2SO_4$

No.	Temp.	Mol. Wt.	Type	Slope
59	19.7	38,700	A	0.85
60	25.2	20,600	C	0.80
61	"	26,800	C	0.79
62	"	21,900	A	0.78
63	"	29,100	C	0.80
64	"	29,700	C	0.79
65	20.5	26,000	B	0.80
66	"	31,100	C	0.72
67	"	26,500	A	0.78
68	33.5	40,000	*	0.74
69	"	34,500	B	0.88
70	"	27,300	B	0.82
71	"	31,100	B	0.85
72	28.8	28,000	B	0.89
73	"	22,900	C	0.79
74	"	35,200	C	0.82
75	"	49,700	C	0.84
76	"	38,300	C	0.81
77	"	44,300	C	0.82
78	"	41,300	C	0.82
82	25.0	20,000	C	0.75

*Balance irregularity - Discard



Legumin (b)

Six runs were made on legumin (b). The observed molecular weights showed the same variation as for legumin (a).

Legumelin

Legumelin is the water soluble pea protein. FA-F curves are shown in figure 12. The equation of the straight line was found to be

$$FA = 0.183 + 0.75\ F$$

The molecular weight is 13,500. Dissociation is obvious in run #100 in figure 12. The initial surface concentration in #100 was 0.17; in #103 it was 0.52 mg/M^2.

The molecular weight data are summarized in table 2. The FAF curves are shown together in Figure 13. The legumin curves are omitted because of their variability. They resemble the vicilin curve, however.

High Pressure Results

High pressure runs were made with the Wilhelmy balance in the pressure range from one to forty dynes. The F-A curves for egg albumin, vicilin, legumin (a), legumin (b) and legumelin are shown in figure 14. The corresponding compressibility curves are shown in Figure 15.

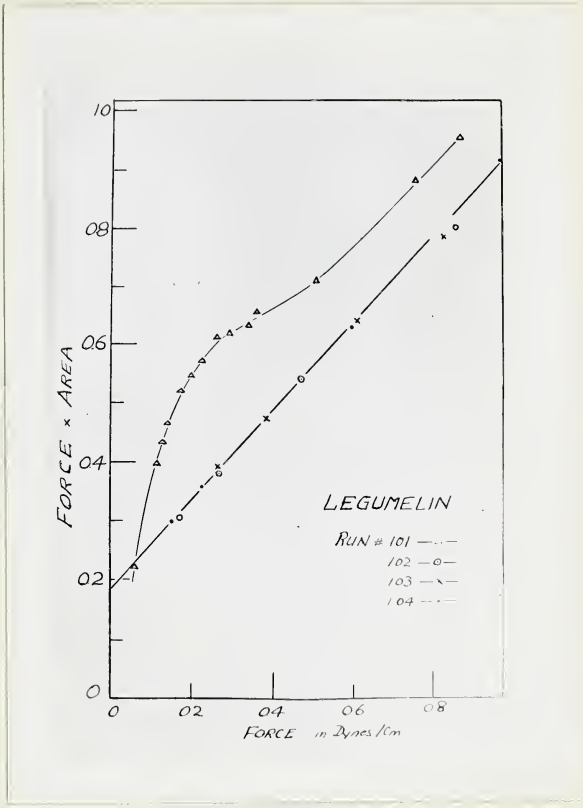


Figure 12.
FA-F Curve for Legumelin

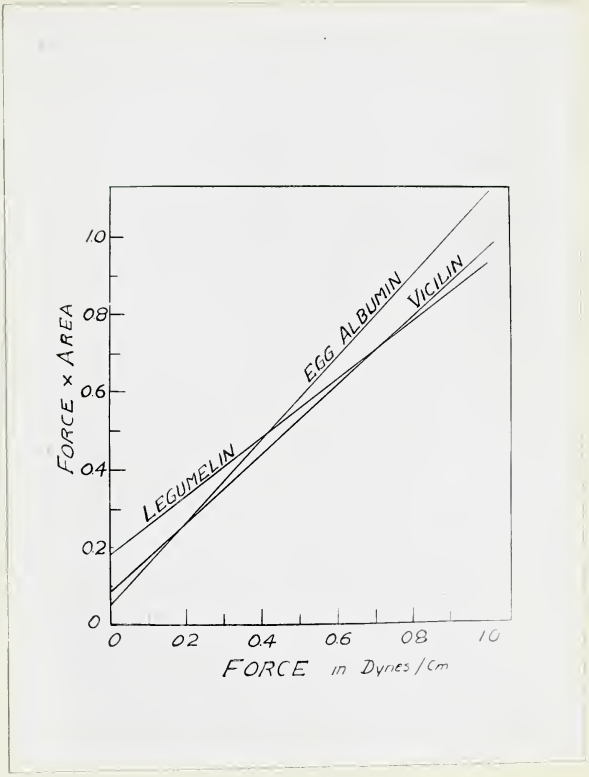


Figure 13.
FA-F Curves

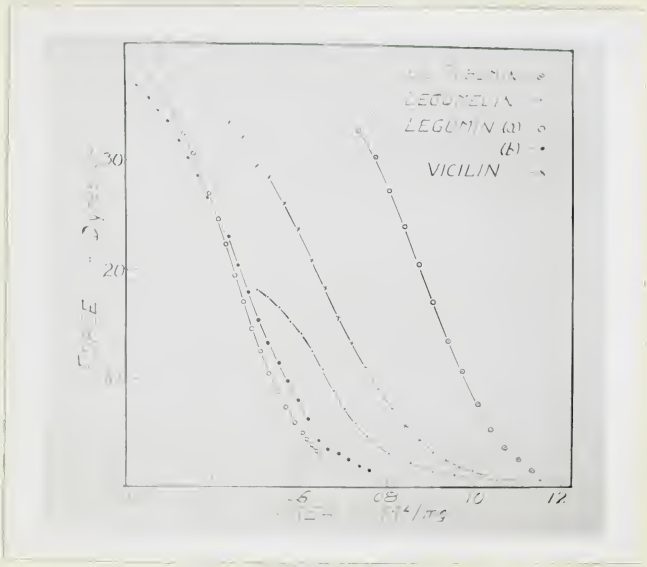


Figure 14.
F-A Curves for pea proteins and
egg albumin

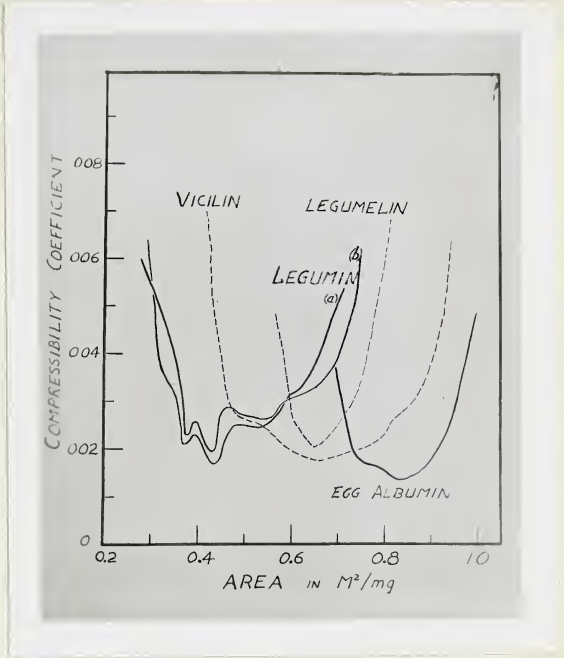


Figure 15.
Compressibility Curves

Surface Potential Results

The surface potential data are somewhat limited because:

1. early in the investigation the FP-54 electronic tube failed, and several months elapsed before a replacement could be obtained,

2. the high humidity interfered with the operation of the polonium electrode. The operation of the electrode is based on the fact that the polonium is radioactive. The emission of alpha particles ionizes the air below the electrode making it locally conducting. When the humidity is high the resistance of the air gap is found to be unusually high. This is probably caused by the absorption of alpha particles by condensed water on the electrode.

Philippi (34) reported similar difficulties. The preparation of a new polonium coat on the electrode did not improve its operation.

The data presented here are believed to be typical.

ΔVA-A values are plotted in Figure 16. It will be recalled that ΔVA is proportional to the vertical component of the apparent dipole moment of the spread molecule. The close relation between ΔVA-A, F-A, and compressibility coefficient curves is shown in Figure 17 for legumin (b).

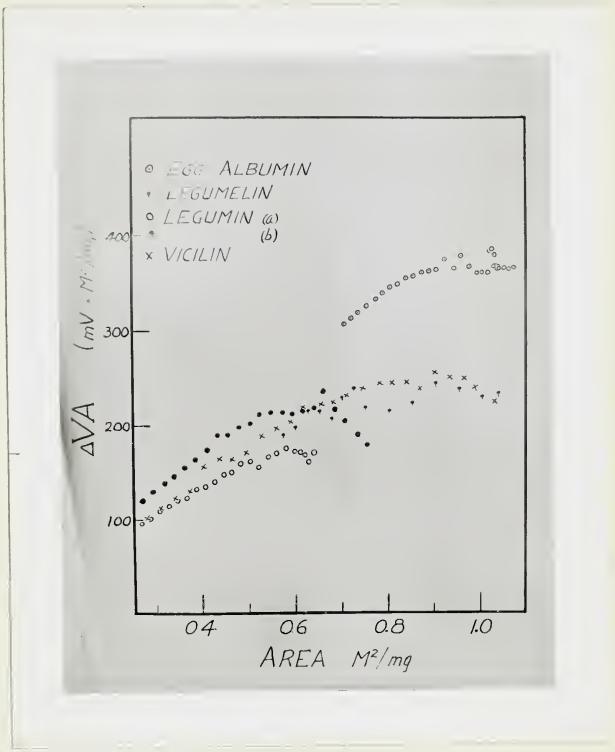


Figure 16.
VA-A plots for pea proteins and egg albumin

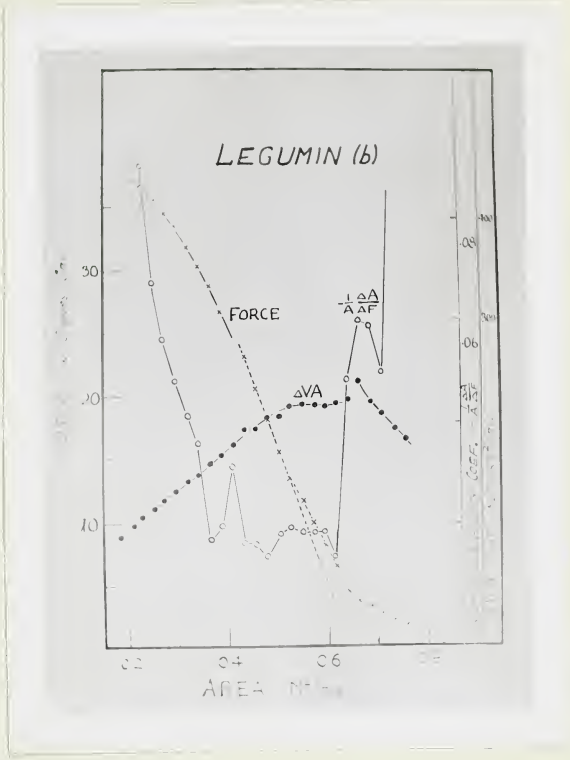


Figure 17.

F-A, ΔVA-A, compressibility -A curves for legumin (b)

Expansion Pattern Results

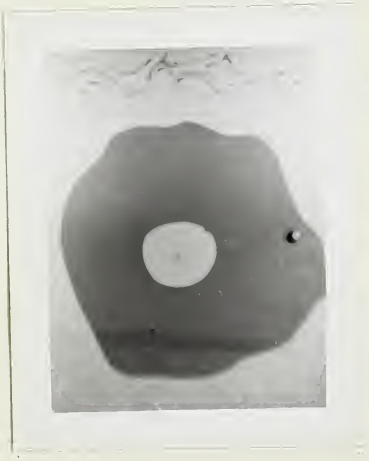
Expansion patterns of legumelin, vicilin and egg albumin were made on water. Legumin did not spread on water. Expansion patterns of all four were made on 35% ammonium sulfate solution. Patterns on the solution were identical in all respects with those on water.

Egg albumin gave a typical star-shaped internal boundary. A similar pattern was observed with legumin and vicilin. Legumelin however gave a perfectly smooth internal and external pattern. Photographs of the expansion patterns are shown in Figure 18.

Figure 18(a) is an expansion pattern of the original pea protein extraction which contained the three proteins, vicilin, legumin and legumelin. A very irregular pattern is obtained. (b) is the pattern for legumelin, while (c) is that of vicilin. Figure 18(d) shows a pattern for egg albumin.

A series of patterns can be obtained from a given protein in which the change from a liquid film to a condensed film is evident. At very low pressure the film for legumin gives a smooth circular pattern. As the pressure is increased the internal pattern is found to be like a multi-pointed star. Patterns at pressures of about one dyne are star-shaped with about three points. This is shown in Figure 18(e) for legumin(a).

(a)



(b)

(c)

Figure 18.
Expansion Patterns

(d)

(e)

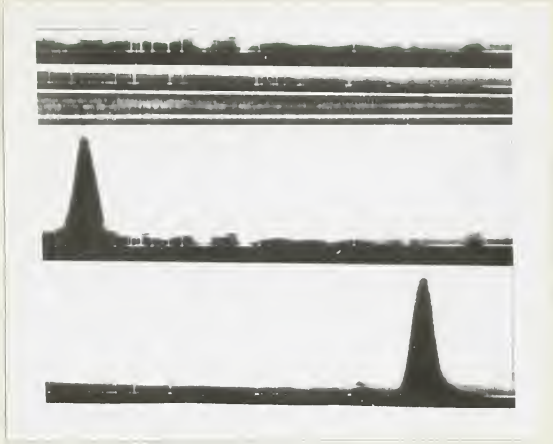
Figure 18.
Expansion Patterns

Electrophoresis Results

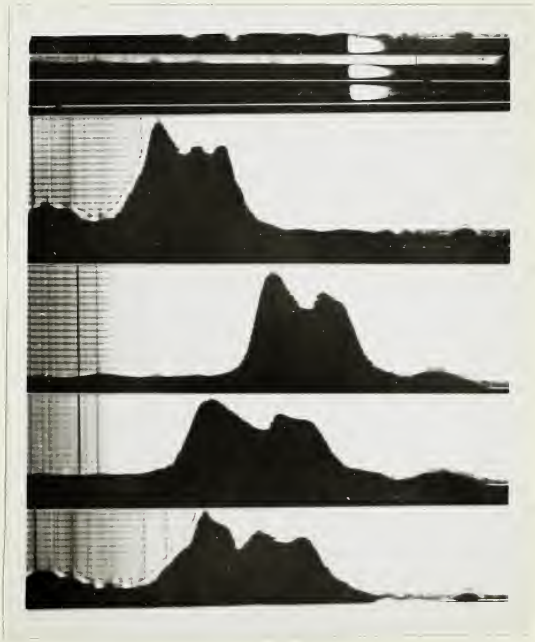
Electrophoresis patterns for vicilin, legumin (a), legumin (b) and legumelin are shown in Figures 19, 20 and 21.

Fig. 19(a)	Vicilin	pH=6.41,	conc. 0.3%,	3% NaCl
20	Legumin(a)	6.75	0.3%	5% NaCl
19(b)	Legumin(b)	7.83	1 %	0.2 N buffer
21	Legumelin	7.73	0.3%	0.2 N buffer

These results are included here through the kindness of Dr. McCalla and Mr. Wetter.



(a) Vicilin



(b) Legumin (b)

Figure 19.

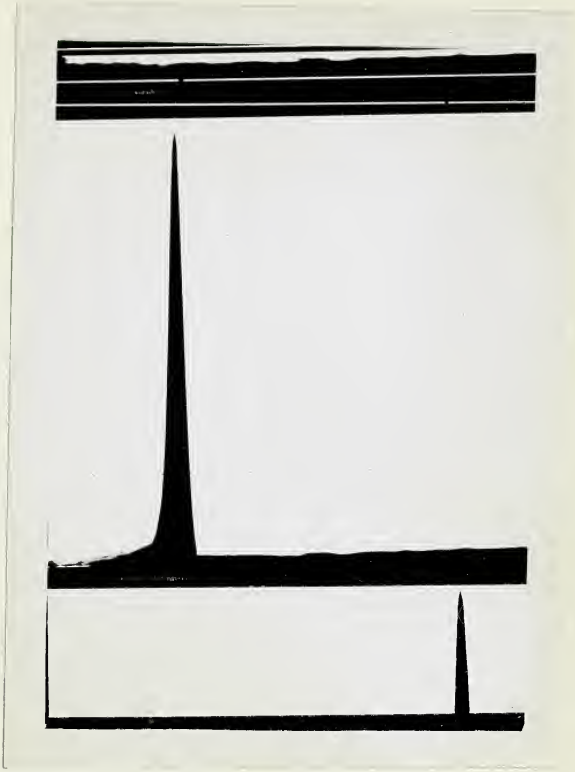


Figure 20.
Legumin (a)

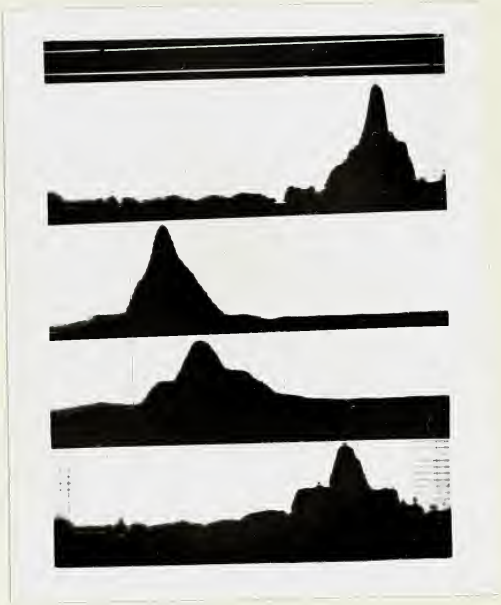


Figure 21.
Legumelin

Table 2

	Egg Albumin	Vicilin	Legumin(a)	Legumin(b)	Legumelin
Molecular Weight	43,200	28,400	20-50,000	20-50,000	13,500
Solvent	H_2O	3% NaCl	10% NaCl	buffer	H_2O
Limiting Area M^2/mg.					
1. F-A extrapolation	1.05	0.86	0.62	0.64	0.78
2. FA-F slope	1.05	0.81	0.75 to 0.89	0.74 to 0.86	0.75
Areas M^2/mg.					
1. Min. Compressibility	0.84	0.65	0.42	0.45	0.65
2. VA-A change i	1.02	0.81	0.62	0.66	0.74
ii	0.86	0.63	0.53	0.53	0.63
Min. Compressibility coefficient	0.013	0.018	(0.018 (0.023	(0.020 (0.025	0.020
Electrophoresis peaks	-	1	1	3	3
Molecular wt. by McConnell	45,800	198,000	-	-	15,600

DISCUSSION OF RESULTS

DISCUSSION OF RESULTS

Gaseous protein films have not been studied extensively. In 1938 Adam (1) wrote "Protein films are coherent; their molecules are much too large for gaseous films to be detectable". In 1945 Bull (8) reported a determination of the molecular weight of egg albumin by measurements on gaseous protein monolayers. Using a Wilhemly film balance he reported pressures as low as 0.04 dynes. For the pressure range 0.04 to 0.4 dynes he found the FA-F points fell on a straight line with the equation

$$FA = 0.0554 + 0.970\ F$$

from which the molecular weight, 44,400, is calculated.

In the present investigation the gaseous film has been studied over the range of 0.01 to 1 dyne using a horizontal film balance. It has been found that under the proper conditions, the FA-F points fall on a straight line with the equation

$$FA = 0.0569 + 1.05\ F$$

from which the molecular weight of 43,200 is calculated.

The 2% difference between these two molecular weight determinations is believed to be of little significance. It is possible that it is due to differences in the methods of determining concentrations. Microkjeldahl nitrogen analysis was used in this investigation whereas Bull determined concentration by drying samples at 105°C. in a

vacuum oven for twenty four hours. Even under such conditions of drying it is possible for some water to remain tightly adsorbed to the protein.

Guastalla (45,46) has reported molecular weight determinations on proteins by means of a horizontal film balance. The molecular weight of egg albumin was found to be about 40,000.

Osmotic pressure measurements for egg albumin have yielded molecular weights of 45,160 by Bull (9), 43,000 by Marrak and Hewitt (26), 45,800 by McConnell (28).

Ultracentrifuge studies by Svedberg and his collaborators yield 44,000 by sedimentation methods and 40,500 by equilibrium methods (5,39).

Molecular weights for two of the three pea proteins have been determined by McConnell (28): vicilin, 198,000; legumelin, 15,600. These results will be discussed in a later section.

Electrophoresis measurements show legumelin to be made up of three components as shown in Figure 19(d). Close examination of the pattern shows that there is one main component accompanied by two minor ones. Legumin (b) in Figure 19(b) is shown to consist of three components in approximately equal proportions. The electrophoresis patterns shown in 19(a) and

20 were made in strong sodium chloride solutions rather than the customary buffer solution. Other work by Wetter has shown anomalous results when sodium chloride solutions were used. It cannot be said definitely that vicilin and legumin (a) are single components as indicated by their patterns.

It is quite probable that legumin (a) is not made up of a single component. Comparison of the data obtained from legumin (a) and legumin (b) indicates they are the same material, because of similar observed molecular weights and similar limiting areas as shown in Table 2.

Compressibility curves are nearly identical and are much different from the curves for the other pea proteins (Figure 15). There was one important difference noted, however. Legumin (a) precipitated when its stock solution of about 0.5% protein concentration was diluted with water. The phosphate buffer solution of legumin (b) was diluted one to fifty, and no precipitation occurred.

Egg Albumin Structure

In the following discussion, the egg albumin measurements are interpreted in terms of molecular structure. Some theories of protein structure are shown to be unlikely, others are supported and extended. Conclusions are drawn about the

mechanism of spreading. In this respect the dissociation hypothesis suggested in an earlier section is enlarged. Behavior of the protein film is used to interpret the structure of the spread molecules.

Bergmann (6) suggested that proteins are made up of long polypeptide chains. Wrinch (40) developed the cyclol theory based on the assumption of a lactim-lactem transformation involving two amino-acid residues, which are supposed to form a closed hexagon. Pauling (50) and Palmer (48) have suggested a layer type structure in which a continuous polypeptide chain is the fundamental unit of the molecule.

Much evidence to support the Pauling structure has been reported in the literature. Since the results of this investigation also support it, the subsequent discussion of the structure of egg albumin will be based on it.

The Pauling-Palmer layer-type structure for the egg albumin molecule postulates a long polypeptide chain folded in such a manner that there are four layers. Each layer consists of about eight segments of the chain lying side by side, each segment having about twelve amino acid residues in it. Such a layer is about forty angstroms square. Since the amino acid residues are pictured as being perpendicular to the surface and alternating above and below it, the thickness of the layer is about ten angstroms. Pauling suggests that the chain

segments are held in position through hydrogen bonding between the nitrogen atom in the peptide linkage and the oxygen of the carboxyl group of the nearest alpha amino acid in the neighboring segment. The egg albumin molecule is considered to be made up of four layers stacked one on top of the other with the same polypeptide chain throughout. Such a molecule would be a cube, forty angstroms on the edge, containing about 384 amino acid residues.

Detergent studies have led Palmer (48) to suggest that one side of each layer is predominantly polar while the other side is non-polar. This view is supported by Shaw (52) who has made surface area measurements on egg albumin with the standard Brunauer-Emmett adsorption apparatus. He found abnormally high surface areas for water vapour. He attributes his results to the adsorption of water vapour at the polar interfaces between layers. X-ray studies on denatured egg albumin show that egg albumin consists of long polypeptide chains (42,43,44).

In the present investigation, the area of the surface covered by gaseous protein particles has been measured. From Table 2 the observed area is

$$1.05 \ M^2/mg.$$

which corresponds to

$$\frac{1.05 \times 43{,}200 \times 10^3 \times 10^{20}}{6.06 \times 10^{23}} = 7500 \ \text{Å}^2/\text{molecule}$$

It is generally recognized that protein monolayers are about 10 Å thick. That is, the film is the same thickness as the Pauling-Palmer layer. It seems probable that in the spreading process the four layers are displaced so that they lie on the surface side by side, linked together by the continuous polypeptide chain. If such were the case the area of the surface covered by the molecule would be

$$4 \times 40^2 = 6400 \text{ Å}^2.$$

The small difference between the observed and the calculated area can be readily accounted for. Firstly, there is the carbohydrate prosthetic group to be considered. If it were spread on the surface it would occupy about 3% additional area. Secondly, packing of the layers on the surface is probably much more random than in the native cubic molecule.

Hughes (47) has suggested that the spread protein consists of long polypeptide chains on the surface with the amino acid lying on the surface. Such a structure is incompatible with the area figures presented above because in that arrangement the area of the molecule would be several times the observed figure.

The agreement between the observed and calculated areas indicates that the orientation of the acid residues in

the layers is preserved during spreading. If only the residues on the edge of the layer were to drop down on the surface the dimensions of the layer would be changed from 40 Å square to about 50 Å square. The spread molecule would then occupy about 10,000 $Å^2$. Since the observed area is only 7,500 $Å^2$, such re-orientation seems unlikely.

The findings of Rothen and Landsteiner (51) are of interest here. They prepared an anti-egg albumin rabbit serum and found that the specific antibody in the serum combined with egg albumin monolayers deposited on glass slides. It is believed that the surface configuration of a protein is the controlling factor in a protein-antibody reaction. The Rothen-Landsteiner results indicate that the original surface configuration of the egg albumin is preserved to a large extent in the spread film.

The foregoing discussion has dealt with egg albumin monolayers of the normal type, that is, those which give a C-type FA-F curve. In the section "Experimental Results" a dissociation theory was presented to account for the anomalous A-type FA-F curve. In no other monolayer work has dissociation been reported as such. Guastalla (46) reported a molecular weight of 12,000 for hemoglobin using a very sensitive horizontal film balance. The accepted figure for hemoglobin

is 63,000 based on ultracentrifuge sedimentation measurements (5). Although Guastalla apparently did not mention dissociation to explain his low result, it seems apparent that dissociation had taken place.

It is observed in Figure 10 that little or no dissociation takes place at initial surface concentrations of greater than 0.4 mg./M^2. At that concentration the average surface area per molecule is

$$\frac{43{,}200 \times 10^3 \times 10^{20}}{0.4 \times 6.06 \times 10^{23}} = 17{,}800 \text{ Å}^2$$

The area of the surface actually covered by the molecule is about 7,500 $Å^2$. Below this initial surface concentration dissociation apparently occurs. Some reason must be given to explain why a particle occupying a large area of free surface should dissociate, whereas the same molecule occupying little free surface does not. From a consideration of the free surface energies involved, one can see that the 17,800 $Å^2$ available area is more efficiently covered if the molecule breaks up into smaller particles. The smaller particles would have much higher thermal velocities than a single large molecule and hence would cover a larger area in unit time. In this way the free surface energy of the surface would be reduced to a greater extent by a number of small particles.

Since the four layers are joined together by single peptide links, it is quite possible that one or more of the links could be readily broken. As the total area of the film (protein + bare surface) is reduced the tendency for dissociation to take place is reduced until a point is reached after which no further breakdown takes place. Thus, in the early part of the compression run, the number of particles on the surface is increasing, giving rise to the steep portion of the type A FA-F curve. After the dissociation rate is reduced to zero the number of particles on the surface is constant so the FA-F points fall on a straight line. From the equation of the straight line the average particle weight is calculated. The slope of the straight line is found to be greater than 1.05, quite frequently double that figure. This can be accounted for by postulating

1. that re-orientation of the acid residues takes place during dissociation, thus giving rise to an increase in surface area covered by the protein particles,

2. that it is an effective increase in area due to the increased velocity of the particles.

From measurements on normal gaseous egg albumin films, the surface area covered by the molecules is found to be

1.05 M^2/mg. An extrapolation of F-A curves for condensed films gives a value for the area covered by the molecules when they are closely packed together on the surface. The area so obtained is also 1.05 M^2/mg. The agreement indicates that the orientation of the egg albumin molecule in the condensed state at zero pressure is the same as in the gaseous state. Although this point appears quite significant, it seems to have been overlooked by other investigators.

The point of minimum compressibility is found to occur at 0.84 M^2/mg. It is believed that this is the minimum area the protein film can occupy without collapsing. (Bull (8)) The ΔVA-A curve shows considerable fluctuation in the range 0.84 to 1.02 M^2/mg. Evidently the film is undergoing some fundamental changes in this range. Philippi (32) believes the film is being "dehydrated". The "dehydration" consists of squeezing out of the film the tightly adsorbed water molecules. The surface potential fluctuations indicate that the process is quite irregular, perhaps involving a phase change in the film. It is interesting to note that in the range below the point of minimum compressibility, the ΔVA-A shows a very steady change of potential. If the film were collapsing as suggested by Bull, it would be expected that the vertical

dipole moment of the film molecules would show considerable variation from time to time. Such variations would be caused by the presence of crumped and folded protein film under the electrode. Such variations are not observed; instead, just a steady change of dipole moment is observed. This indicates that "collapse" of the film consists of a steady re-orientation of the molecules in the film.

Summary of the egg albumin structure

The egg albumin molecule is believed to be a cube made up of four layers. When the molecule is spread on the surface the layers "unleaf" and lie side by side on the surface. When the spreading area is very large the loose bonds between layers are broken. The spread molecule, even in the gaseous state, is believed to be highly orientated, having much the same surface configuration as it had in solution. At very high film pressures the orientation of the spread molecules is changed in some regular manner.

Vicilin Structure

In the following discussion a molecular model for vicilin is prcposed on the basis of a layer type molecule. A mechanism of spreading is suggested and supporting evidence presented. A dissociation theory is advanced to explain the differences between monolayer and osmotic pressure molecular weights.

Vicilin is a globulin obtained from peas. The method of preparation, expansion pattern spreading, FA, FA-F, compressibility and ΔVA-A curves show it to be quite different from the other globulin, legumin and the albumin, legumelin. No molecular weight data has been published for vicilin nor for either of the other two pea proteins. No film balance studies have been published on any of these materials.

Film balance work on gaseous vicilin films gives a molecular weight of 28,400. Osmotic pressure determinations by McConnell (28) give a weight of 198,000. Obviously some explanation must be offered to account for these divergent results. The following discussion is based on two assumptions:

1. the vicilin molecule is a layer type molecule as proposed by Pauling (50) for globulins in general.

2. when spread as a monomolecular film, the molecule dissociates so that each layer exists as a separate particle.

The area of the surface covered by gaseous vicilin particles is found to be $0.81\ M^2/mg$.

$$\text{or } \frac{0.81 \times 28{,}400 \times 10^3 \times 10^{20}}{6.06 \times 10^{23}} = 3800\ \text{Å}^2/\text{molecule}.$$

The layer is, then, about 62 Å square. The cubic layer-type molecule should be about 62 Å on the edge. About six layers 10 Å thick, would have to be stacked up in order to form such

a molecule. Then the molecular weight of the cubic molecule would be

$$6 \times 28{,}400 = 170{,}000.$$

If seven layers were used, the molecular weight would be

$$7 \times 28{,}400 = 199{,}000$$

The molecular weight of vicilin in solution was found to be 198,000.

Unfortunately all protein concentration determinations in the monolayer investigations were made by microkjeldahl nitrogen analysis, while all osmotic pressure protein concentration measurements were made by the residue weight method as used by Bull. A rough check of methods showed that the figure 28,400 was somewhat low in terms of a concentration based on residue weights. Regardless of the uncertainty in the concentration figures, the evidence presented above strongly suggests that in solution the vicilin molecule is a cube made up of six or seven layers. When the vicilin molecule is spread the layers separate completely and the observed molecular weight is found to be one-sixth or one-seventh of the molecular weight in solution.

The dissociation of vicilin during spreading is apparently rapid and complete. The initial surface concentration was varied over a very wide range, 0.26 to 1.05 mg/M^2, but no

appreciable variation in molecular weights was observed. Evidently the particle with the molecular weight of 28,400 is the basic unit of the vicilin molecule in solution.

It is evident from Table 2 that vicilin is much like egg albumin in that the gaseous particles are highly orientated and the limiting areas, areas of minimum compressibility, and areas at which the vertical dipole moment dhanges are all interrelated. It should be noted, however, that the absolute areas (M^2/mg) for vicilin are somewhat smaller than the corresponding egg albumin areas. This may be explained by two factors:

1. It will be recalled that the egg albumin molecule is made up of four units tied together. In any random arrangement of four units a considerable "dead" space exists for each molecule. For this reason the effective area of the molecule may be increased over the calculated area.

2. The vicilin particle on the surface must be either thicker than the ten angstroms reported for most films, or the polypeptide chain segments must be closer together. It is rather unlikely that the film is thicker, because the thickness of the film depends on the length of the amino acid residues. It is improbable that vicilin and the other pea proteins are made up predominantly of long amino acids.

Thus, experimental results suggest that the chain segments are more closely packed in the vicilin layers than is the case with egg albumin.

Summary of vicilin structure

The vicilin molecule is believed to be a cube somewhat larger than the egg albumin molecule. The cube is made up of six or seven layers which separate completely during spreading. Like egg albumin, vicilin is highly orientated on the surface even at very low pressures.

Legumin Structure

Legumin is the name applied to the second pea globulin. Preparations were made by two different methods yielding purified products designated as legumin (a) and legumin (b).

It has been shown that legumin (a) and legumin (b) are nearly identical (page 44). The following discussion will be concerned with the globulin "legumin". It will be shown that the molecule is quite large in solution and the structure of the molecule is probably not of the layer type.

According to the crude and somewhat artificial system of protein classification developed in the last century, an albumin is a protein soluble in water. A globulin is a

protein insoluble in water but soluble in dilute solutions of neutral salts. Most albumins are found to have small molecular weights while globulins have larger molecular weights. It is believed that the difference in solubility is caused to a considerable extent by the size of the molecules. Electrical properties of the protein molecule surface are also believed to be important (34). The idea of interaction between the surface charges and the neutral salts is used to explain why globulins are soluble in dilute salt solutions.

The egg albumin molecule is small and is soluble in water. The vicilin molecule is large and soluble in 3% NaCl. The legumin molecule can be peptized only with 10-20% NaCl. The solution so formed is probably only a colloidal suspension, because most of the legumin can be centrifuged down at ordinary speeds. This indicates that the legumin molecule is probably larger than the vicilin molecule.

From a consideration of the shape of the compressibility curves in Figure 15 it is evident that legumin is quite different from egg albumin and vicilin.

It is evident from the molecular weight determinations that dissociation is taking place during spreading. The variability of the observed molecular weights is shown in Table 1. As pointed out earlier, the type A and C curves show

the same distribution, indicating that dissociation has taken place in both cases. It is believed that dissociation is complete in a type C run before readings are made, while dissociation is still in progress when the initial readings were made in a type A curve.

The temperature at which the monomolecular runs are made seems to have little or no effect on the observed molecular weight. The initial surface concentration of the legumin seems to have some effect; for example in runs 76, 77 and 78 where the concentration was high, the observed molecular weights were high.

A comparison of surface areas covered by the gaseous film and the condensed film shows no agreement (Table 2). This indicates that the gaseous and condensed molecules are quite different. The larger area for the gaseous molecule is probably a result of acid residues lying on the surface or the presence of a thick condensed film. Since the area range for gaseous molecules includes the figure for gaseous vicilin molecules, it seems more likely that the gaseous molecule resembles vicilin. In that case the condensed legumin film must be thicker than the condensed vicilin film.

Legumin did not spread on distilled water. All the other proteins did.

It is apparent that legumin is quite different from egg albumin and vicilin. The properties of egg albumin and vicilin have been shown to be directly related to a layer type molecule. It is reasonable to suggest then that the legumin molecule is not a layer structure.

No osmotic pressure measurements were made on this material. From the electropheresis patterns shown in Figure 19 b, it is evident that legumin consists of three components in about equal proportions. The properties of the monolayer are average properties. For instance, to determine molecular weights, one counts the number of particles on the surface by means of an FA-F plot and obtains a number average molecular weight. The variation observed here cannot be attributed to the possibility that there are three different kinds of molecules on the surface.

Summary:

The legumin molecule is probably larger than the vicilin molecule. The layer type structure for the legumin molecule seems unlikely.

It is possible that the Wrinch cyclol structure would be adequate; the experimental results do not support or reject it.

Legumelin Structure

Legumelin is the water soluble pea protein. The molecular weight is found to be about 13,500. McConnell reported a molecular weight of about 15,600. Since osmotic pressure results are usually higher than monolayer results, the agreement is considered satisfactory. 13,500 is the lowest* particle weight determined by the film balance in this investigation. A low molecular weight is expected from a consideration of the legumelin expansion pattern, Figure 18 b. The smooth circular type of pattern exhibited suggests that the film is liquid or gaseous at a pressure of one dyne. To be liquid or gaseous at that pressure, the film must be made up of small particles. All the other proteins studied here have star-shaped patterns obtained from larger molecules compressed into a condensed film.

At low initial surface concentrations the legumelin is found to dissociate into about twice as many particles, as shown in Figure 12.

Electrophoresis patterns show that the legumelin solution contained one major and two minor components.

The low pressure, high pressure, and electrical properties are found to be similar to those for egg albumin. One distinctive point is that the limiting areas are low

* Obtained from type C FA-F curves.

compared with the other proteins studied here. Since the areas are expressed in units of M^2/mg. it appears that the packing of the polypeptide chains in the legumelin molecule must be much closer than in egg albumin. In other words the molecule appears to be denser. Apart from this, it is evident that the legumelin structure is much the same as the layer type egg albumin structure.

SUMMARY

SUMMARY

It has been shown that the monolayer technique can be applied to the study of proteins with reasonable success. It has also been shown that the study of proteins is not a one-man task. Information must be gained from all possible sources and then correlated to develop a complete understanding of protein structure. X-ray data, electrical data such as dipole moments discussed by Cohn and Edsell (11), electron microscope observations and ultracentrifuge determinations must be used in addition to the osmotic pressure, electrophoresis, and monolayer measurements described herein. Some of the data is conflicting, but much of it is not. Some of the conflicts observed are due to the very nature of the proteins. Being a fundamental part of living matter, they are very complex and subject to change. The term "molecular weight" is probably a misnomer in that it implies that proteins are composed of clearly defined units, and that the units of a particular substance are all the same. It has been shown that a protein which apparently is a single chemical compound may appear to be made up of three separate species in electrophoresis analysis. The sizes of the particles are found to be dependent on the treatment given the protein. Who is to day what the molecular weight of a particular substance is, when investigators

with equally precise methods find widely different "molecular weights"? Does the protein molecule exist in the living organism as a single unit? Probably the molecules are held together by weak chemical, electrical and physical bonds.

Plant proteins seem to be less clearly defined than animal proteins. This is shown in part by the failure of most plant proteins to crystallize. The instability of pea proteins during spreading also demonstrates this.

Because of the fact that the term "molecular weight" is artificial and somewhat misleading it is felt that "particle weight" would be a better expression.

The numerical data for the five proteins studied in this investigation are summarized in Table 2.

It has been shown that egg albumin is probably a layer type molecule which is "unleafed" in spreading. The molecule is found to dissociate if the spreading area is very large. The molecular weight from gaseous monolayer measurements is found to be about 43,200.

Much evidence is presented to show that vicilin is also a layer type molecule. A particle weight of 28,400 determined by monolayer methods is shown to be compatible with a particle weight of 198,000 determined by osmotic pressure measurements.

Legumin is shown to have much the same properties when prepared by two different methods. The molecule is apparently larger than the vicilin molecule in solution. The structure of the molecule is probably not of the layer type; possibly the Wrinch cyclol theory would fit.

Legumelin is found to have a very small particle weight. Probably the structure is of the layer type.

SUGGESTIONS FOR FURTHER WORK

The protein monolayer project is far from completed.

1. Determinations of molecular weight and degree of spreading may be carried out on different substrates, at various pH's and at various temperatures.

2. The effect of temperature gradients across the surface may be studied in great detail.

3. The thermodynamics of monolayers is very interesting (16). Work along this line would involve making monolayer runs over a wide range of temperatures. The thermostating system would have to be extended to permit measurements over a wide range, say, from -30°C. to 70°C.

4. The effect of ultraviolet light falling on the spread film may be studied.

5. Isoelectric points of proteins may be determined by spreading methods.

6. The surface potential studies may be extensively expanded.

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